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DNA methylation at enhancer regions: Novel avenues for epigenetic biomarker development

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1. ABSTRACT

Biomarkers are molecules or features which can provide clinically-relevant information about a particular disease state, thus providing useful tools for oncologists. Recently, a number of studies have demonstrated that DNA methylation holds great promise as a novel source of cancer biomarkers. Although promoter regions have been the focus of most investigations thus far, mounting evidence demonstrates that enhancer sequences also undergo extensive differential methylation in cancer cells. Moreover, enhancer methylation correlates with target gene expression better than promoter methylation, providing unexplored strategies for biomarker development. Here, we review important considerations associated with the clinical analysis of DNA methylation at distal regulatory regions. Notably, we highlight emerging literature addressing the methylation status of enhancers in development and cancer, and subsequently discuss how enhancer methylation can be exploited to guide disease management. While acknowledging current limitations, we propose that the methylation state of enhancer regions has the potential to headline the next generation of epigenetic biomarkers.

2. INTRODUCTION

In recent years, our understanding of the molecular mechanisms underlying malignancy has increased vastly, leading to the identification of numerous biomarkers that are currently employed in the clinic (1). A biomarker refers to any molecule or characteristic which can be measured and accurately provide information about the status of a given physiological or pathological process (2). Biomarkers are extremely valuable tools for clinicians as they can guide disease management throughout the cancer continuum – from initiation to progression (3). Notably, biomarkers are used to assess cancer risk, ensure early detection, determine prognosis, and predict response to therapy (3). However, there are still a large number of clinical situations in which the development of novel biomarkers would greatly improve patient care while reducing treatment-associated costs (4-6). Emerging studies have identified biomarkers which are epigenetic in nature, i.e. that do not rely on mutations in the underlying DNA sequence (7,8). Epigenetic regulation is conferred by a complex network of reversible chemical modifications on DNA and histones which define the transcriptional competency of surrounding *loci* (9). In human cancers, numerous epigenetic alterations are known to drive tumor initiation and progression, which has led to the development of a new class of biomarkers based on chromatin alterations (10-12).

Currently, most epigenetic biomarkers involve the detection of aberrant DNA methylation since the

techniques use for its detection are highly quantitative and thus well suited for clinical use (13). DNA methylation at CpG dinucleotides was first described in 1975 (14,15) and since then a growing body of literature has demonstrated that the transcriptional effect of DNA methylation heavily depends on the genomic sites at which it occurs (16). For example, CpG island methylation in promoter regions correlates with transcriptional silencing of corresponding genes while methylation within gene bodies typically associates with actively transcribed *loci* (16,17). Cancer cells often feature global hypomethylation accompanied by promoter-specific hypermethylation of tumor suppressor genes (18,19). To date, almost all DNA methylation-based biomarkers have focused on methylation occurring in promoters regions, with the most notable example being the methylation status of the *MGMT* gene promoter which is used to predict whether glioblastoma patients will benefit from temozolomide (7). However, recent evidence demonstrates that CpG methylation within enhancer regions also greatly influences chromatin organization during malignant transformation and progression (20,21), uncovering novel opportunities for epigenetic biomarker development.

Enhancers are relatively short (20-400 bp) DNA sequences that bind tissue-specific transcription factors and can regulate transcription at distant *loci* through chromosome looping (22). While most enhancers are found within intergenic regions, a significant number of enhancers can also be found at intragenic sequences (23,24). Active enhancers interact with master transcription factors, a process which is intricately dependent on the enhancer methylation status (25). Hypomethylation of enhancer DNA allows transcription factor binding and thus is typically associated with transcriptional activation of target genes (25). Conversely, inactive enhancers tend to display higher levels of DNA methylation and usually result in silencing of target genes (26). Increasing evidence suggests that these processes are disrupted in cancer cells, leading to cellular de-differentiation (27). Despite the fact that most epigenetic biomarkers solely address promoter status, mounting evidence demonstrates that methylation status of enhancer regions correlates better with target gene expression than promoters (20). Thus, since altered methylation at enhancer sequences represents a unifying feature of tumor cells, it may be exploited as a biomarker at different stages of cancer management.

In this article, we address the unexploited potential of enhancer DNA methylation as a novel class of cancer biomarker. First, we summarize key mechanistic concepts relating to methylation of enhancer sequences and discuss how they relate to transcriptional regulation in development and cancer. These experimental findings will serve as the basis for proposing novel applications of enhancer DNA methylation within the context of cancer biomarkers, which will be discussed in light of their current limitations. Finally, we outline possible therapeutic implications of enhancer DNA methylation and how they contribute to the burgeoning field of personalized oncology. Overall, we integrate recently described evidence to provide a framework for the future use of enhancer methylation in guiding treatment decisions.

3. ENHANCER DNA METHYLATION

In parallel with recent technological advances, mounting evidence demonstrates that DNA methylation is highly dynamic and that its epigenetic effect strongly depends on its particular genomic context (28,29). Importantly, it has become clear that the methylation status of enhancer regions greatly influences cell type-specific gene expression programs (30). While the mechanisms through which enhancer methylation is coupled to transcriptional regulation are not fully understood, some important concepts have emerged from genome-wide studies (20). Interestingly, dynamic modulation of enhancer methylation is observed throughout embryonic development, correlating with differentiation into specific lineages (31). In human cancers, many enhancers become dysregulated, leading to cellular de-ifferentiation and acquisition of malignant properties (32,33).

3.1. General Mechanisms

While the interdependence between enhancer activity and DNA methylation remains under investigation, key mechanistic insights have delineated principles of DNA methylation at enhancer regions. At these genomic sites, DNA methylation usually correlates with enhancer silencing and repression of target genes while active enhancers are typically hypomethylated (30). Active, demethylated enhancers are characterized by the presence of H3K4me1, H3K27ac, p300, pol II, TET1, and enhancer RNA (eRNA) transcription (26,34, 35). Importantly, enhancer activation correlates with nucleosome absence, adding another layer to the complex epigenetic regulation of distal regions (33). Conversely, inactivation and loss of nucleosomes at enhancer regions are followed by *de novo* methylation, thus allowing reversible enhancer methylation in response to environmental cues (36). H3K4me1 is a commonly used marker of active enhancers, and results from the activity of the MLL3/MLL4 methyltransferases and their associated complexes (37). Adding to the complexity of enhancer regulation, it has also been shown that enhancer activity and fidelity is controlled by Polycomb-dependent H3K27me1 and H3K27me2 (38). While the exact mechanism remains incompletely resolved, it is now well accepted that many factors regulate enhancer activity, and that cellular context influences epigenetic regulation at distal enhancers (30).

A critical feature of enhancers is that their activity is highly tissue-specific, and increasing evidence suggests a tight interplay between active enhancers and binding of lineage-specific transcription factors (39,40).

This finding was first reported by Saluz *et al*. almost 30 years ago who demonstrated that enhancers regulated by estrogen receptor (ER) underwent demethylation upon treatment with estradiol, and that enhancer hypomethylation correlated with increased expression of ER target genes (25). More recently, it was also shown that CpG demethylation at glucocorticoid receptor (GR) binding sites correlates with cell type-specific chromatin accessibility and that DNA methylation at the core GR-responsive elements can interfere with GR binding *in vitro* (30). Importantly, emerging data suggests that enhancer activity is also coupled to the epigenetic status of promoters, with active enhancers harboring H3K4me3 or H3K27ac marks at promoter regions, suggesting that transcription factor-induced looping can have long range effects on promoter status (32,40). The intricate relationship between transcription factors and enhancers is further complicated by the recent finding that RNA transcripts can be transcribed from active enhancer regions, giving rise to enhancer RNAs (eRNAs) (41,42). Beyond merely reflecting activation of associated enhancer loci, eRNAs have been attributed multiple functional roles that are critical for optimal target gene regulation (43,44).

From a mechanistic standpoint, a key question that has generated a lot of interest recently is how enhancers regulate transcription at distant *loci* (45). It is important to recognize that the answer takes root in the three-dimensional structure adopted by DNA. Given the flexible nature of the double helix, genomic regions that are linearly far away may be brought together via looping of DNA segments (46). This process of looping can therefore bring enhancers in close proximity to the promoter of their target genes. Since active, demethylated enhancers are bound by factors that promote transcription, spatial proximity to target promoters also stimulates gene expression (47). Given the value of identifying specific enhancer-target gene pairs, methods to quantify direct interactions between any two genomic regions have been developed. Most of them rely on the principle of chromosome conformation capture (3C) (48), which has now been adapted for genome-wide throughput using techniques such as Hi-C (49). This has allowed the generation of 3D maps of chromatin loops, which have revealed that the genome is preferentially distributed within regions of the nucleus called topologically associated domains (TADs) (50). Thus, enhancers should be seen as inherent parts of a complex epigenetic machinery that operates in a three-dimensional fashion.

3.2. Normal Cellular Differentiation

Since enhancer activity defines cellular differentiation, these genomic regions typically represent the most differentially methylated sequences between tissues of distinct lineages (23). Thus, enhancer methylation plays critical roles during embryonic development. As ES cells differentiate, enhancers of pluripotency are inactivated and those linked to differentiation are activated upon dynamic hydroxymethylation of enhancer DNA (51). For example, during neural differentiation, FOXA1-dependent enhancers are intrinsic cell-type-specific regulatory regions of which activities have to be potentiated by FOXA1 through induction of an epigenetic switch inducing DNA demethylation (52). Moreover, differences in DNA methylation between human neuronal and glial cells are concentrated in enhancers and non-CpG sites (53). These data are consistent with the strong epigenetic nature of cell differentiation during embryonic development and highlight the critical role of dynamic enhancer DNA methylation in this process.

In addition to its implication in embryogenesis, enhancer methylation continues to be critical for numerous physiological processes that occur throughout adulthood. In order to maintain their terminal differentiation, cells must actively maintain lineage-specific enhancer activity through enhancer hypomethylation to ensure proper homeostasis (31,34). For example, recent studies have shown that the tissue-specific expression of SF-1/Ad4BP relies on the methylation status of specific distal regulatory regions (54). In addition, dynamic regulation of enhancer methylation is also required in cells that undergo changes in cell fate under physiological conditions such as hematopoeisis. For instance, genome-wide methylation profiling in different T cell lineages demonstrated extensive methylation differences particularly at enhancer regions (55). Likewise, analysis of the DNA methylome and transcriptome in granulopoiesis revealed that differentially methylated sites across different myeloid cells were enriched in enhancer regions (56). Taken together, these findings suggest that enhancer DNA methylation regulates differentiation in normal cells. Thus, enhancers of tissue specific-genes must remain unmethylated and active to maintain cellular identity. Accordingly, misregulation of this process can lead to malignant transformation.

3.3. Human Cancers

In parallel with the dynamic role of enhancer methylation during development, aberrant methylation of distal regulatory regions also characterizes cancer cells of both solid and hematological malignancies (33,57). While promoter methylation has been extensively studied in the context of cancer, mounting evidence suggests that enhancer methylation is likely to be equally or more important to tumor biology. Pioneering studies by Aran *et al*. have demonstrated that expression of target genes correlates better with enhancer methylation than with promoter methylation in multiple solid tumor types (20). Moreover, the effect of methylation differences on gene expression is significantly higher at enhancer sites compared to promoters (20). There is also evidence reporting that cancer-associated enhancer activation is

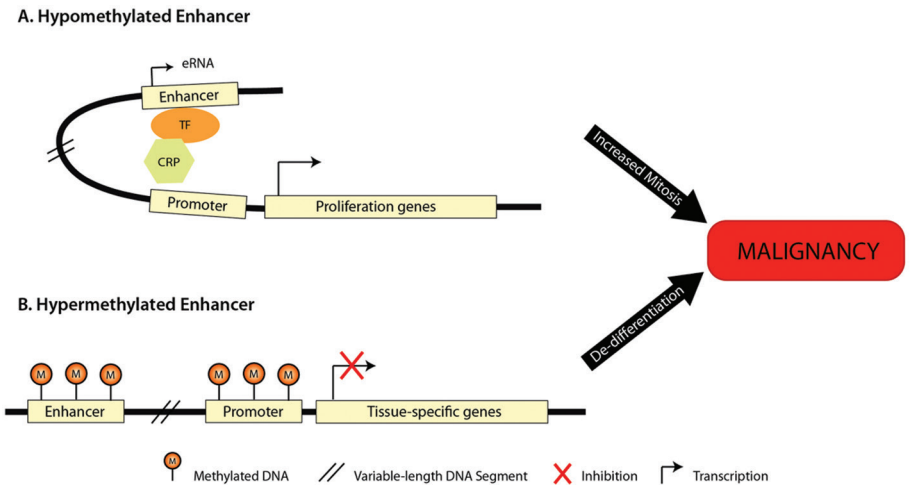

Figure 1. Molecular model of enhancer methylation in human cancers. Hypomethylated enhancers are typically permissive for chromosomal looping and can stimulate the expression of distal proliferation genes, leading to elevated mitotic rate. Iternatively, hypermethylation of tissue-specific enhancers may result in silencing of genes specifying cellular identity, thus promoting de-differentiation.

intricately linked to the acquisition of specific chromatin marks such as H3K4me3 or H3K27ac at promoter regions (32). These data are consistent with a model in which enhancer DNA methylation regulates the epigenetic status of associated promoters and target genes. In cancer cells, differentially methylated enhancers are bound by a larger number of transcription factors than predicted for the genomic average and unmethylated sites contain more transcription factor binding sites than methylated sites, in line with the idea that DNA-binding factors reprogram enhancers in a methylation-specific manner (21). Furthermore, enhancer sites frequently altered in human tumors are evolutionarily conserved (58), suggesting that these regulatory regions are relevant in controlling cancer-associated gene expression.

In tumor cells, the target genes affected by enhancer hypermethylation and hypomethylation regulate subsets of genes with distinct biological properties (21) (Figure 1). Recent evidence demonstrates that many hypomethylated enhancers are shared across a wide variety of tumor types while hypermethylated enhancers are mostly restricted to one cell type (20). Based on this principle, hypermethylation of these tissue-specific enhancers would induce de-differentiation of normal cells into malignant cells. In contrast, the genes regulated by hypomethylated enhancers are preferentially involved in active proliferation and are shared across many tumor types (20). Thus, both hypo and hypermethylated enhancers may provide distinct but complementary information relating to tumor biology. Interestingly, emerging evidence suggests that the methylation state of specific regulatory regions can be inherited transgenerationally (59). Genetic variants associated with specific enhancer methylation sites have been shown to correlate with disease incidence (60). Such variant *loci* are termed methylation quantitative trait *loci* (meQTLs) and their status may be used to determine cancer susceptibility (61), adding another dimension to the use of enhancer methylation in cancer management.

4. ENHANCER METHYLATION AS A NOVEL BIOMARKER

Given the emerging evidence demonstrating that enhancer regions undergo differential methylation in tumor cells, we propose that their methylation status may be exploited to provide relevant disease-related information, thereby giving rise to a new class of epigenetic biomarkers. Over the past decade, key technological advances have led to a dramatic rise in the throughput and sensitivity of DNA methylation analyses, accompanied by a significant decrease in associated costs (1,62). As a consequence, biomarkers based on DNA methylation are becoming increasingly attractive, particularly given our growing knowledge of how the epigenome affects tumor biology. For the first time, we outline some clinical scenarios in which biomarkers based on enhancer methylation may improve current standard of care. It is important to note that significant challenges must be overcome before the routine use of enhancer-based biomarkers is included into clinical practice. However, given the exponential increase in epigenomic technology observed during the past decades, the strategies discussed below provide novel avenues that are likely to become feasible in a reasonably near future.

4.1. DNA methylation analysis

Paradoxically, the DNA methylome present at enhancers displays less intertumoral heterogeneity than the mutational landscape of human tumors (32), making this epigenetic mark more conducive to biomarker development. Adding to its value as a biomarker, DNA methylation represents a stable chemical mark at room

temperature and its degradation is expected to be minimal in clinical storage conditions (63). Importantly, DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues is suitable for DNA methylation analysis (64). An important advantage of DNA methylation-based biomarkers is that cell-free DNA is present in a number of body fluids and thus can be isolated through non-invasive means. To date, researchers have been able to successfully detect and quantify methylation of DNA originating from multiple body fluids including plasma/serum (65), urine (66), sputum (67), and saliva (68). As the list of body fluids containing detectable DNA continues to grow (69), the number of opportunities to develop DNA methylation-based biomarkers also expands.

Currently, DNA methylation analysis methods can be classified in three main categories based on their general mechanism of action: enzyme digestion, antibody/protein enrichment, and bisulfite modification (70). Additionally, each technique can also be coupled to other experimental techniques depending on the question of interest (71). First, since DNA methylation occurs only on cytidine bases, enzymatic restriction of DNA allows the discrimination between methylated and unmethylated DNA at restriction sites (72), serving as the basis for techniques that quantify DNA methylation using restriction enzyme-based assays (72). Secondly, another emerging technique to analyze DNA methylation, methylated DNA immunoprecipitation (MeDIP), relies on the recognition of methylated DNA fragments by an antibody specific to 5-methylcytidine (73,74). The immunoprecipitated DNA can subsequently be analyzed either in a locus-specific or genome-wide fashion depending on the particular question of interest (71,75). Recently, the Infinium HM450 arrays have been highly popular for DNA methylation profiling, and they provide the advantage of covering a number of annotated enhancers (76). Lastly, techniques relying on chemical conversion of DNA are becoming the preferred method for analyzing DNA methylation (77). Typically, DNA templates are subjected to sodium bisulfite treatment, which converts non-methylated cytidines into uracil while leaving methylated bases unaffected, thereby providing a means to identify the methylation status of specific genomic sequences (78). Among bisulfate-based techniques, Sequenom EpiTYPER has been well cited in the literature as a platform for DNA methylation analysis (79). Finally, DNA methylation profiling may subsequently be analyzed via the UCSC genome browser to identify whether relevant differentially-methylated regions (DMRs) reside within enhancer sequence. Since enhancer regions have been annotated, they have been integrated into computational packages that can identify whether a particular sequence resides within an regulatory region (80). Thus, DNA methylation can be quantified through various methods that offer a broad range of sensitivity and specificity.

While there are a wide variety of DNA methylation analysis platforms available, a number of factors must be considered when selecting a method that fits optimally within the context of clinical use. Important variables include genome coverage (global vs. loci), area studied (CpG islands vs. CpG poor regions), types of data (quantitative vs. sensitive), and availability of DNA (paraffin-embedded samples vs. high purity DNA), all of which influence the choice of an optimal DNA methylation detection techniques. For example, while restriction enzyme-based methods are able to resolve methylation differences in low CpG density regions, the sensitivity of these assays is limited to enzymes' target sites (81,82). In addition, they require substantial amounts of DNA with high purity and integrity, which is not always available in a clinical setting. In contrast, MeDIP allows genome-wide coverage but does not provide base pair resolution. Currently, the "gold standard" in DNA methylation analysis involves bisulfite conversion after which the resulting DNA undergoes PCR amplification and sequencing (83). An important advantage of these techniques is that it requires less DNA while providing base pair resolution. Methylation-specific PCR (MSP) is another technique requiring chemical conversion that provides information at the single-*locus* level. MSP does not require sequencing and is independent of the use of methylation-sensitive restriction enzymes (84). Pyrosequencing, on the other hand, is a method that relies on quantifying bioluminometric signals generated followingrelease of pyrophosphates during nucleotide incorporation (85). This assay is flexible and can be used to quantify methylation in CpG islands as well as CpG poor regions. Given that many bisulfite-based methods continue to improve as the technology evolves, DNA methylation as a biomarker will also become more viable for clinical testing as time progresses.

4.2. Cancer predisposition

Previous studies have evaluated the link between genetic variants with cancer risk and have overwhelmingly revealed that cancer incidence cannot be explained solely on the basis of genetic inheritance. Interestingly, a large number of cancer-related SNPs have been found within regulatory sequences (86). Unfortunately, because they are often found in intergenic regions, the SNPs are often overlooked and thus a number of these potentially relevant SNPs are not exploited. Recent data strongly suggest that these intergenic GWAS hits should be further investigated as they may represent key elements in transcriptional regulation (87,88). Providing novel insights into this issue, an increasing number of SNPs have been linked to specific DNA methylation patterns (meQTLs) in cancer, offering a potential mechanism to mask the effect of disease-related SNPs (60). A growing number of meQTLs are being linked to many disorders outside of cancer, including type 2 diabetes (T2D) and other complex diseases (89,90). Interestingly, meQTLs have also been discovered in studies involving twins, suggesting that meQTLs are important determinants of disease predisposition (91). Thus, meQTLs may be

used to identify individuals at higher risk of developing malignancies, and therefore likely to benefit from an earlier and more frequent cancer screening regimen.

In line with this idea, known breast cancer-associated risk *loci* have been reported to associate with enhancer sites whose methylation status preferentially correlates with intertumor variation. These included estrogen receptor (ER) enhancers whose methylation levels significantly correlated with cyclin D1 and MAP3K1 expression in ER+ breast cancer cells (21). Further studies have shown that correlation of target gene expression was higher for enhancer methylation than for SNPs (21). In colon cancer, differentially methylated enhancers are enriched in haplotype blocks that contain genetic variants associated with colon cancer incidence (32). Recently, Heyn *et al*. provided a comprehensive meQTL catalog containing DNA methylation associations for 21% of interrogated cancer risk polymorphisms spanning 13 solid cancer types (61). A key finding was that epigenetic risk alleles occur independently of tissue type, which draws attention to the stable character and potential function as risk epi-polymorphisms. Overall, 72% of meQTLs were also detected in paired normal tissues, suggesting that relevant meQTLs could be detected in tissues that do not require invasive sample collection (61). Overall, these data indicate that DNA methylation at enhancer regions interacts with clinically relevant SNPs and therefore influences cancer predisposition, a phenomenon that may be exploited clinically.

4.3. Early detection

It is well appreciated that tumors evolve over time and consequently that detecting the disease at an early stage increases the likelihood of favorable patient outcome (92). Currently, the use of biomarkers for screening and early detection is restricted only to a few cancer types despite strong evidence demonstrating that cancer screening leads to better medical and economic consequences (1). The exponentially decreasing cost of DNA methylation analysis makes the assessment of enhancer methylation an attractive option for large-scale screening programs (93). In addition, since DNA can be extracted non-invasively from numerous body fluids, the population may be more likely to follow this screening regimen as opposed to more invasive screening methods such as a colonoscopy. As previously introduced, enhancers that become hypomethylated in cancer cells tend to regulate genes associated with distinct biological functions compared to hypermethylated enhancers. Notably, enhancers that become hypomethylated in cancer tend to regulate genes promoting cellular proliferation (20). Importantly, many of these differentially-methylated enhancers are shared across many different cancer types (20), suggesting the presence of a conserved epigenetic program underlying neoplastic transformation. The methylation status of these enhancers could thus be used as an early indicator that a tumor may be present and that further diagnostic tests should be considered. Along similar lines, these biomarkers could also helpful in monitoring the presence of recurrent disease after therapy. While these recurrently hypomethylated enhancers still require extensive validation prior to clinical use, current evidence suggests that analyzing their methylation status provides an attractive opportunity to develop a new generation of affordable and non-invasive screening biomarkers.

Another avenue that may lead to earlier detection is the identification of specific genetic alterations that disrupt enhancer regions and lead to their aberrant activity. For example, Northcott *et al*. have shown that somatic structural variants juxtapose the normally repressed GFI1B oncogene proximal to an active enhancer in medulloblastoma, leading to aberrant GFI1B expression (94). Importantly, these genetic variants were specifically observed in medulloblastoma subgroups 3 and 4, for which a driving alteration had not yet been described (94). While few cases like these have been reported to date, recent advances in genomic sciences suggest that more of these enhancer alterations are likely to be discovered. Since multiple body fluids contain DNA suitable for sequencing, such pathologic variants could also be detected in body fluids and provide an early indication that a tumor is likely present. Thus, as we characterize more of these enhancer-regulating aberrations, the dynamic interplay between the genome and the epigenome may also be exploited in the context of cancer biomarkers.

4.4. Diagnosis, prognosis and treatment response

Demethylated and active enhancers are largely unique to individual cell types. Since enhancer methylation levels are intimately linked to cell differentiation, differentially methylated enhancers may also provide information regarding the differentiation status of tumors. As such, the gain of methylation at tissue-specific enhancers may indirectly serve as a measure of cellular differentiation. In general, less differentiated tumors tend to be more metastatic and aggressive compared to more differentiated neoplasms (95), thus differential enhancer methylation may help determine patient prognosis. Hu *et al*. recently investigated genome-wide methylation changes in renal cell carcinoma (RCC) (96). Their results indicate that altered methylation was particularly enriched in kidney-specific enhancers, and was correlated with downregulation of target genes (96). Furthermore, many differentially methylated enhancer regions were enriched in transcription factor binding sites, particularly those associated with hypoxia, a key feature of RCC (97). Importantly, hypermethylation of those enhancers correlated with lower overall survival in both univariate and multivariate analyses (96). These novel findings suggest that the relationship between hypermethylation

of specific enhancers and prognostic features may represent a widespread feature of human cancers that could be exploited in biomarker development.

Currently, the clinical diagnosis of human tumors is made by taking into account a number of factors that may vary from one cancer type to the other. For example, breast tumors can be subdivided on the basis of histological and molecular findings into distinct clinical entities such as ER/PR+, HER2+, luminal A/B, and triple negative breast cancer (TNBC) (98). However, even within these subtypes there exists considerable molecular heterogeneity such that the clinical course is highly variable from one patient to the other (98). Thus, since the methylation status of key enhancers can greatly influence cancer-specific gene expression (21), analysis of enhancer DNA methylation may improve the establishment of a diagnosis and also a prognosis even within well-established clinical subtype. Along the same lines, integrative genomic and epigenomic studies may be conducted to identify combinations of specific enhancer activation/loss and somatic gene mutations that can accurately and reproducibly provide relevant clinical information (99), a concept which can be adapted to many tumor types. Moreover, we speculate that enhancer DNA methylation could also be useful for patients diagnosed with a genetic cancer syndrome, who must often live with the fear that a tumor may arise at any time (100). For some of these patients, it may take years before cancer appears, and some may even live cancer-free all their life. If we can identify enhancers whose methylation state can predict the likelihood that these patients can develop cancer at different stages of their lifespan, it would greatly reduce anxiety and improve quality of life in this patient population (100). This would also stimulate research into the epigenetic implications of familial cancer syndromes, an aspect that has often been overlooked.

Another difficult clinical problem to address is determining the primary site of a metastatic lesion with unkown origin. In these particular cases, very little is known about the molecular features driving progression, thus treatment remains difficult to select and often fails (101). To address this problem, enhancer DNA methylation may be used to help identify the origin of the primary tumors. A recent study conducted in colorectal cancer has shown that inactivated enhancers are highly crypt-specific whereas those who gain active enhancer marks are relatively noncrypt-specific, suggesting that enhancer status can discriminate between tumors originating from different cell types (32). Thus, tumors retain an epigenetic “fingerprint” of their tissue of origin at enhancer regions. Accordingly, the enhancer methylation profile of the metastases could be compared with signatures of normal cell types to infer the most likely organs from which the tumor has originally spread. The information provided by enhancer methylation could therefore allow clinicians to request more specialized tests to identify the primary tumor with more accuracy, which would lead to improved diagnosis and more appropriate treatment selection.

In recent years, the number of FDA-approved targeted therapies has continued to dramatically rise (102). However, the rate at which biomarkers predicting response to the agents has not followed this trend, such that a number of new drugs are given to an unselected population of patients because of the lack of appropriate biomarker (103). As a result, there is a wide range of responses to these treatments, which significantly hampers clinical management. For example, the use of antiangiogenic drugs has been successfully implemented in the clinic for some years now, but remains without a clear biomarker to predict whether a given tumor may be intrinsically resistant or sensitive to these therapies (104). Given recent reports linking neoangiogenesis and aberrant DNA methylation, it is probable that the enhancer methylome may hold promising candidate biomarkers to predict response to antiangiogenic agents (105-107). In parallel, there is an emerging interest in developing novel immune-based therapies, and there are currently very few molecular biomarkers available to determine which patients are likely to benefit from these treatments, which are often very expensive (108). Finally, we propose that biomarkers based on enhancer DNA methylation hold tremendous promise in numerous diseases outside of cancer. Since some of the most common diseases in the world such as heart disease (109) and diabetes (110) have a strong epigenetic basis, it logically follows that the enhancer methylome may emerge as a key source of next-generation biomarkers.

5. THERAPEUTIC IMPLICATIONS

It is now widely accepted that genetic and epigenetic aberrations drive the initiation and progression of human neoplasms (111). However, the inherent reversibility of the epigenome, as opposed to static mutations occurring within the DNA sequence, makes it more amenable to therapeutic manipulations (112). In line with this idea, many antagonists of epigenetic regulators have shown success in clinical testing for a number of tumor types (113). Moreover, inhibitors of DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) are currently in clinical use for some haematological malignancies (114-116). All this evidence suggests that cancer cells hijack the epigenetic machinery to promote their survival and tumorigenic properties. Given that enhancer activity plays critical roles in regulating the cancer epigenome (117), abnormal enhancer methylation may therefore be exploited in the development of novel therapeutic strategies.

Along those lines, one potential approach represents the use of compounds targeting the enzymes

catalyzing the addition of methyl marks at enhancer regions, the DNA methyltransferases (DNMTs). As previously mentioned, tissue-specific enhancers become hypermethylated in transformed cells, leading to loss of tumor suppression and normal differentiation. This implies that the lost gene function of these lineage-specific genes can potentially be reinstated by simply reversing the abnormalities in DNA methylation. Currently, azacytidine and decitabine are the two most widely used FDA-approved demethylating drugs (118). Despite the efficacy of these drugs in haematological neoplasms, their pleiotropic effects and incompletely understood mechanism-of-action indicate that their activity may be related to methylation changes in enhancer regions. Another exciting development has been the recent discovery that a significant fraction of tumor-suppressive microRNAs (miRNAs) becomes epigenetically silenced in various neoplasms (119,120). Since enhancer methylation often dictates the transcriptional competency of target genes, hypomethylating agents may lead to the re-induction of these miRNAs, thereby hampering tumor growth (120,121).

As previously mentioned, enhancer activation involves an intricate interplay of various macromolecules including a novel class of non-coding transcripts derived from active enhancers which are termed eRNAs (122,123). Stimulus-dependent transcription of eRNA itself has been associated with transcriptional activation of nearby coding genes, suggesting it that eRNAs actively regulate expression of target genes (124-126). For example, eRNA production from p53-bound enhancer regions was shown to be required for efficient p53 transactivation (127). Given the widespread alterations in enhancer methylation observed in tumor cells, this enhancer-eRNA interdependence may be therapeutically exploited in cancer. Accordingly, RNA-interfering agents such as antisense oligonucleotides and small interfering RNAs (siRNAs) may be employed to degrade selected eRNAs, which in turn attenuates enhancer-mediated transcriptional activation of target oncogenes. This strategy has been previously employed in a pioneering study which showed that antisense RNA-mediated degradation of DNA methyltransferase mRNA significantly inhibited tumorigenesis (128). Recently, functional studies have demonstrated that targeted eRNA-degradation via siRNAs resulted in specific inhibition of target gene-regulation from the associated enhancer sites (127,129). The successful depletion of eRNAs via RNA-interference highlight the potential of such epigenetic therapies in human cancers and provide the rationale for further investigation of their clinical efficacy (96).

The epigenome is inherently regulated by cues originating from the extracellular microenvironment through the activation of specific signaling pathways that converge into chromatin remodeling (130). To date, the molecular signals that initiate the methylation and/or demethylation of enhancer regions have not been elucidated. However, as our understanding of the interplay between cellular signaling and epigenetics improves, it might become possible to predict the relative activation state of specific signaling pathways based on the methylation profiles of critical enhancers. This therapeutic strategy would thus couple metabolism and epigenetic regulation, an emerging connection in the field of cancer biology (131). The potential of this approach has been recently demonstrated in neuroblastoma, where inhibition of CDK7 attenuated the downstream tumor-promoting effect of n-Myc activity at enhancer regions (132). Interestingly, this strategy was particularly successful in tumor cells with *MYCN* amplification, suggesting that therapeutic strategies based on enhancer dysregulation could be targeted to patient populations harbouring specific genetic alterations (132). As such, enhancer DNA methylation represents valuable molecular information that should be integrated within the framework of personalized cancer therapy.

6. CONCLUSIONS

In this article, we build on recently uncovered concepts in transcriptional regulation to propose that the DNA methylation status at enhancer regions may reflect many aspects of tumor biology and provide useful information in disease management. Importantly, we highlight that DNA is a very stable molecule that can be harvested from a number of body fluids and that quantification of DNA methylation is increasingly accurate and sensitive, which are key features of effective biomarkers. In addition to these technical advantages, mounting evidence demonstrates that enhancer DNA methylation correlates better with expression of cancer-related genes than promoters, thus representing an improvement over current biomarkers based on promoter methylation. Specific subsets of enhancers preferentially undergo differential methylation in different contexts, thus providing a wide range of potential biomarkers to help clinicians assess cancer risk, detect cancer early, and define patient prognosis. Overall, we believe the information provided by enhancer methylation will not only help guide disease management but also stimulate research into novel frontiers in biomarker development.

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